PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/70	A1	(11) International Publication Number: WO 91/10747
		(43) International Publication Date: 25 July 1991 (25.07.91)

(21) International Application Number: PCT/US90/06675

(22) International Filing Date: 31 October 1990 (31.10.90)

(30) Priority data:
466,061 16 January 1990 (16.01.90) US

(71) Applicant *(for all designated States except US):* PRUTECH RESEARCH DEVELOPMENT PARTNERSHIP II [US/US]; R & D Funding Corp., 1290 Ridder Park Drive #1, San Jose, CA 95131 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* SENN, Donald, E. [US/US]; 10146 Maxine Street, Ellicott City, MD 20143 (US). WIER, Marjorie [US/US]; 6277 Sunny Spring, Columbia, MD 20146 (US). ZIMMERMAN, Daniel, Hill [US/US]; 5527 Oakmont Avenue, Bethesda, MD 20817 (US).

(74) Agent: JACOBS, Seth, H.; Davis Hoxie Faithfull & Hapgood, 45 Rockefeller Plaza, 28th Floor, New York, NY 10111 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE, SE (European patent), SU, US.

Published
With international search report.

(54) Title: COMBINATION ASSAY FOR ANTIBODY OR ANTIGEN

(57) Abstract

An immunoassay for determining a first antigen (Ag_1) and/or an antibody (Ab_2) to a second antigen (Ag_2) in a sample, which immunoassay comprises the steps: (a) contacting the sample with a solid support on which Ag_2 and an antibody (Ab_1) to Ag_1 have been coated so that sample Ag_1 and sample Ab_2, if present, will become bound to Ab_1 and Ag_2, respectively; (b) adding Ag_2 in the form of a detecting reagent; the addition being performed under conditions allowing the formation of an immune complex between added Ag_2 and sample Ab_2 bound to the solid support in step (a); (c) adding Ab_1 in the form of a detecting reagent; the addition being performed under conditions allowing the formation of an immune complex between added Ab_1 and sample Ag_1 bound to the solid support in step (a); (d) measuring the amount of immune complex formed in step (b) and (c) and taking the amount as an indication of the amount of Ag_1 and/or Ab_2 in the sample. All or part of the antigenic entitie(s) in Ag_2 coated on the solid support and/or Ag_2 used as the detecting reagent are derived from different sources. In an alternative mode Ag_2 and Ab_1 in the detecting reagent is equipped with biotin so that the immune complexes formed in steps (b) and (c) can be measured by the use of a biotin binding substance.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**ES**	Spain	**MG**	Madagascar
AU	Australia	**FI**	Finland	**ML**	Mali
BB	Barbados	**FR**	France	**MN**	Mongolia
BE	Belgium	**GA**	Gabon	**MR**	Mauritania
BF	Burkina Faso	**GB**	United Kingdom	**MW**	Malawi
BG	Bulgaria	**GN**	Guinea	**NL**	Netherlands
BJ	Benin	**GR**	Greece	**NO**	Norway
BR	Brazil	**HU**	Hungary	**PL**	Poland
CA	Canada	**IT**	Italy	**RO**	Romania
CF	Central African Republic	**JP**	Japan	**SD**	Sudan
CG	Congo	**KP**	Democratic People's Republic of Korea	**SE**	Sweden
CH	Switzerland			**SN**	Senegal
CI	Côte d'Ivoire	**KR**	Republic of Korea	**SU**	Soviet Union
CM	Cameroon	**LI**	Liechtenstein	**TD**	Chad
CS	Czechoslovakia	**LK**	Sri Lanka	**TG**	Togo
DE	Germany	**LU**	Luxembourg	**US**	United States of America
DK	Denmark	**MC**	Monaco		

COMBINATION ASSAY FOR ANTIBODY OR ANTIGEN

Background of the invention

1. Field of the invention:

This invention relates to a combination immunoassay for detection of an antigen and an antibody. The format of the antigen part of the assay is antibody:antigen:antibody where the antigen is the analyte. The format of the of the antibody part of the assay is antigen:antibody:antigen. The invention particularly relates to a combination immunoassay for hepatitis B surface antigen (HBsAg) and/or antibody to human immunodeficiency virus (HIV-1 and/or HIV-2).

2. Description of the Prior Art.

Blood banks currently employ two separate tests to screen donated blood for the presence of antibody to HIV and HBsAg. To screen blood for antibody to HIV, an enzyme linked immunoassay is used in which a complex is formed consisting of HIV antigen coated onto a solid support, human antibody to HIV from the sample, and anti-human antibody coupled with horseradish peroxidase. To screen for HBsAg, a sandwich type assay is commonly employed in which the sandwich is formed of antibody to HBsAg, HBsAg from the sample, and antibody to HBsAg coupled with horseradish peroxidase.

It would be desirable to test for HBsAg and antibody to HIV, as well as for other combinations of antigens and antibodies, in a single assay. In order to do so, however, the specificity and sensitivity of separate assays must be maintained while combining all the components of two assays onto a single assay on one solid support, such as a microtiter plate or a bead. A product which successfully accomplishes this is not currently available.

EP-A-173,295 describes a process for simultaneously screening for HBsAg and antibody to HIV. The process disclosed consists of incubating a sample with a solid support coated with purified HIV and polyclonal anti-HBsAg; adding detecting probes consisting of biotinylated monoclonal antibody to HBsAg and biotinylated antibody to human antibody; then adding strepavidin conjugated to horseradish

peroxidase and substrate to develop a color. However, the HIV part of this format is similar to that used by blood banks currently which requires dilution of the sample tested.

In order to effectively test for both HBsAg and antibody to HIV, a neat (undiluted) sample should be employed. For testing a neat sample for antibody to HIV an assay format must be used wherein HIV antigen is both coated on the solid support and used as the detecting reagent. This format eliminates the dilution required when using an assay that includes a labeled anti-human antibody as described above.

When testing a neat sample, however, it is difficult to achieve both the desired sensitivity and specificity. Neat samples contain a high concentration of materials which may non-specifically react with components of the assay, leading to false positive results

There are several ways in which to address this problem in a test for antibody to HIV alone. For example, the HIV antigens may be purified to remove contaminating substances which contribute to non-specific interactions. Alternatively, different source antigens may be employed so that the specific antigen of interest is the predominant common antigen. Another possibility is to add non-HIV antigens from the source of the HIV material in order to complex with antibodies in the sample which would otherwise bind non-specifically to the assay components and generate a non-specific signal.

Different sources of reagents in a sandwich type assay for one analyte have been used in the past in order to eliminate non-specific binding. For example, Belanger et al., Clinica Chimica Acta, 48 (1973), pp.15-18 discloses a sandwich type assay for alpha fetoprotein in which antibody derived from goat is coated on a solid support, and a rabbit antibody to alpha fetoprotein is used as the labeled reagent. The use of antibodies from different sources reduces the non-specific signal otherwise generated when the material on the plate and the labeled reagent are from the same source. For example, where an antibody on the solid support is from the

same animal as the labeled antibody, then the antibody on the plate may non-specifically bind to material in the sample, which then non-specifically binds to labeled antibody, generating an undesired signal. Where a different animal source is used to produce the antibodies then it is less likely that the material which is non-specifically bound to the plate will also bind to the labeled antibody. The reason for this is that it is unlikely that the antibody which bound the material to the plate will also be found in the labeled reagent. Since the antibodies were derived from different animal species, they differ in affinity, avidity and non-specific interaction with the non-specifically bound material. Also, if the sample contains species specific antibody to either the capture antibody or the detection antibody, elevated non-specific signals will not occur, provided the antibodies are from different sources.

Assays employing the antigen:antibody:antigen-format with antigens derived from different sources have previously been described (EP-A-313,986; US priority from 1987 and EP-A-307,149; GB priority from 1987).

SUMMARY OF THE INVENTION

Prior to the above-mentioned 1987 US priority date the usefulness of the different source antigen concept was recognized.

It has now been found that using antigens from different sources in a combination immunoassay of the type given above may lead to an increase in the sensitivity and specificity in testing for an antigen in the other part of the combination assay. This finding allows a neat or minimally diluted sample to be effectively tested for both anti-HIV antibody and HBsAg, simultaneously. The combination assay achieved allows both antigen and antibody to be tested for in a single assay using only one sample, thereby conserving sample and reducing the number of assays required. Similarly, it has also been found that by using biotinylated antigen and biotinylated antibody a valuable combination assay for an antibody and an antigen, respectively, can be accomplished, allowing a reliable determination of the

presence of an antibody specific to the biotinylated antigen or the presence of an antigen reacting with the biotinylated antibody.

In particular, a combination immunoassay for determining the presence, absence, or amount of a first antigen or an antibody to a second antigen has been found which comprises the steps:

a) contacting the sample with a solid support on which the second antigen and an antibody to the first antigen have been coated so that said first antigen and antibody to said second antigen, when present in the sample, will become bound to (complexed with) their immunological counterparts, respectively, coated on the solid support;

b) adding second antigen in the form of a detecting reagent for antibody bound by the second antigen coated on the solid support; the addition being performed under conditions allowing the formation of an immune complex between said added second antigen and antibody specific to said second antigen bound to the solid support in step (a);

c) adding antibody to said first antigen in the form of a detecting reagent for first antigen bound by the antibody coated on the solid support; the addition being performed under conditions allowing the formation of an an immune complex between said added first antibody and first antigen bound to the solid support in step (a);

d) determining the presence, absence, or amount of the immune complex formed in step (b) and (c) in order to determine the presence, absence, or amount of the first antigen and/or the antibody to the second antigen in the sample,

wherein the second antigen coated on the solid support contains antigen that is derived from a first source and the second antigen used as a detecting reagent contains antigen that is derived from a second source, which first and second sources are different.

The antigen(s) coated on the support and the antigen(s) in the detecting reagent may originate from different cell

lines, or one may originate from a cell line and the other may be chemically synthesized, or derived by recombinant genetic engineering techniques.

The second antigen coated on the support and/or present in the detecting reagent may be a mixture of antigens differing with respect to the source from which they derive. Such mixtures used in the invention may contain different relative amounts of antigens from a given source. The antigen on the support may be completely derived from one source, whereas the antigen in the detecting reagent may be a mixture of antigens derived from two or more sources, or the other way round.

According to a specific aspect of the invention an assay is provided which screens for both HBsAg and antibody to HIV. In this mode of the invention the first antigen is hepatitis surface antigen and the second antigen is HIV antigen.

A further mode of the present invention is a combination immunoassay for determining the presence, absence, or amount of a first antigen or an antibody to a second antigen which comprises the steps:

a) contacting the sample with a solid support on which the second antigen and an antibody to the first antigen have been coated so that said first antigen and antibody to said second antigen, when present in the sample, will become bound to (complexed with) their immunological counterparts, respectively, coated on the solid support;

b) adding as a detecting reagent biotinylated second antigen under conditions allowing the formation of an immune complex between said biotinylated second antigen and antibody specific to said second antigen bound to the solid support in step (a);

c) adding as a detecting reagent biotinylated antibody to said first antigen under conditions allowing the formation of an immune complex between said biotinylated antibody and said first antigen bound to the solid support in step (a);

d) determining the presence, absence, or amount of the immune complex formed in step (b) and (c) by

d1) addition of a biotin-binding substance labeled with a marker to complex with the biotinylated reagents bound to the solid support in step (b) and (c), and

d2) detecting the presence, absence, or amount of the complex between biotin and the biotin-binding substance on the solid support in order to determine the presence, absence, or amount of the first antigen and/or antibody to the second antibody.

This latter mode of the present invention is particularly useful for combination assays determining antibody to HIV and hepatitis virus antigen, i.e. assays in which the first antigen is a hepatitis antigen (e.g. HBsAg) and the second antigen is HIV antigen. A preferred embodiment is that the biotinylated second antigen and the second antigen coated on the solid support are from different sources (see above).

The steps (b) and (c) in the embodiments given above may be performed simultaneously by mixing the detecting reagent for antibody to second antigen with the detecting reagent for the first antigen, e.g. by premixing the HIV antigen and the hepatitis antigen. Steps (a), (b) and (c) may often be followed by one or more washing procedures prior to a subsequent step.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The details of the general inventive concept will now be presented with reference to a combination assay for HBsAg and antibody specific to HIV . The principles outlined can be applied to the generalized forms of the invention as presented above.

The term "HIV antigen" in this specification refers to a polypeptide or protein which has the same sequence or partial sequence as that of a native HIV, or is immunologically cross reactive with native HIV. The term thus encompasses haptens which have this immunological property.

The term "HIV" refers to HIV-1 and HIV-2 and any other human immunodeficiency virus that might be discovered in the future.

The term "detecting reagent" is used herein to refer to a reagent which is added to form an immune complex with sample analyte bound to the solid support. An example of a detecting reagent for antibody to HIV is a conjugate of HIV antigen and a label (marker), such as horseradish peroxidase. Another example is an HIV antigen which is biotinylated (biotin being the label). The term may also refer to an antibody to the first antigen, for example an antibody to HBsAg that may or may not be conjugated with a label. In case the detecting reagent contains no label or the label is a group exerting bioaffinity against a certain counterpart (ligand), the complex formed between the detecting reagent and sample analyte bound to the solid support is further complexed with, for example, a labeled ligand that binds by bioaffinity to the detecting reagent. Such a labeled ligand can be anti-goat antibody conjugated to an appropriate enzyme such as horseradish peroxidase where the detecting reagent is an antibody of goat origin. If the detecting reagent is an antibody, it preferably is of different origin from the one coated on the solid support, for instance one may be a goat antibody while the other is a mouse antibody.

Different cell lines refers to cell lines derived from different species or from different individuals of the same species. The cell lines in question must have the ability to express the antigen in question, e.g one or more HIV antigens.

In one preferred embodiment, an HIV infected cell line is used as a source for producing HIV antigen coated on the solid support and a different HIV infected cell line as a source for the detecting HIV antigen. Suitable cell lines are well known in the art and readily available. Preferred cell lines are HIV infected human T-cell lines H9, MOLT-3, CEM and HSB. HIV antigen from infected cell lines is also commercially available in lysate form from Pharmacia Diagnostics Inc., DuPont Biotech Inc., and Organon Teknika.

In another preferred embodiment, recombinantly derived HIV antigen(s) from a defined host is used in one HIV antigen, but not both. Bacterial protein impurities in a recombinant HIV reagent prepared using an E. coli host will not result in a non-specific signal where the same bacterial impurities are not present with both HIV antigens. Recombinant HIV antigen(s) prepared in an insect host, particularly gp160, is commercially available from Repligen Corp (Cambridge, Mass., U.S.A.). The preparation of recombinant HIV antigens, including using an E. coli or Bacillus host, is known in the art.

In another preferred embodiment one cell line is an HIV infected cell line such as H9 or MOLT 3 and the other cell line is a recombinant host cell line. Similarly, one cell line can be a recombinant host cell line, such as an E. coli cell line, and the other a different recombinant host cell line, such as the insect cell line Spodoptera frugiperda.

In a different embodiment, either HIV antigen coated on the solid support or HIV antigen used as a detecting reagent contains one or more HIV antigens that are chemically synthesized. Conventional polypeptide synthesis techniques, for example Merrifield synthesis, can be used. See for instance EP-A-267,802; 247,557; 284,587; 278,148; 231,914 and 246,829 and WO-A-8702775 and US-A-4,735,896 and 4,833,072.

HIV antigen coated on the solid support or HIV antigen used as a detecting reagent may be a mixture of HIV antigens from different sources. Thus, in a preferred embodiment, the mixture may contain an HIV antigen from an infected cell line combined with a recombinantly produced HIV antigen. The recombinantly derived HIV antigen may then restore antigenic determinants that have been removed in part during the preparation and purification of an HIV antigen from the infected cell line. HIV antigens that are part of the mixture may be present in both the detecting reagent and HIV antigen coated on the solid support. For example, HIV antigen coated on the solid support may be derived from an HIV infected cell line, and the detecting reagent may contain a recombinantly or synthetically produced HIV antigen combined with a further

purified HIV antigen derived from an HIV infected cell line and vice versa.

In a further preferred embodiment, one of the HIV antigens coated on the solid support or used as the detecting reagent is a combination of a recombinant HIV antigen from a defined host, for example insect cells, and an HIV antigen from an infected cell line. The other remaining HIV antigen may then be a recombinant HIV antigen from a different defined host, for example E. coli, which may also be combined with an HIV antigen from an infected cell line. The infected cell line employed can be the same one for both the solid support and the detecting reagent. For example, the HIV antigen coated on the solid support may be a mixture of a recombinant HIV antigen from one host, such as insect cells, and a purified HIV antigen from the infected cell line H9, while the detecting reagent may contain a mixture of a recombinant HIV antigen from another source, such as E. coli and an HIV antigen from infected cell line H9. The preferred recombinant HIV antigen is gp160.

HIV antigen can be in a mixture of many antigens or a single purified antigen. HIV-1 antigens such as p17, p24, p31 gp41, gp51, p55, gp120, p24, p66 and gp160 and corresponding HIV-2 antigens can be employed alone or mixed together. In a preferred embodiment HIV antigen in form of a viral lysate prepared from HIV infected cells is used.

By selecting appropriate purified HIV components the assay can be tailored to test only for particular antibodies. In order to test specifically for antibody to p24, whole purified lysate can be coated on the solid support and pure p24 added as the detecting agent for antibody to HIV antigen. Specific testing for other viral components may be performed in a similar way.

By selecting HIV-1 or HIV-2 antigens as the coating on the solid phase and as the detecting reagent, antibodies to HIV-1 or HIV-2, respectively, will be detected. By the proper combination of HIV-1 and HIV-2 antigens antibodies reacting with only one of the antigens as well as antibodies reacting with both of them will be assayed simultaneously. For

instance synthetic HIV-2 specific peptides are known (e.g. US-A-4,182,556) and can be combined with lysate of HIV-1 infected cells.

The detecting reagent is preferably biotinylated forms of HIV antigen and/or antibody to HBsAg. This means that step (d) above comprises reacting the complex formed in steps (b) and (c) with a biotin-binding substance labeled with a marker so that a complex is formed between biotin on the solid support and said biotin-binding substance, which latter complex subsequently can be measured due to the presence of the marker. Examples of biotin-binding substances are anti-biotin antibody, strepavidin and avidin.

Biotinylation of both HIV antigen and antibody to HBsAg can be accomplished by conventional means. Anti-biotin antibody is preferred and can be of polyclonal or monoclonal origin and is available from standard commercial sources.

The antibodies to HBsAg can be either monoclonal or polyclonal and are commercially available or can be produced by standard techniques, for instance by immunization of guinea pigs, goats, or sheep with highly purified HBsAg or production of monoclonals by immunization of mice followed by fusion of antibody producing plasma cells with immortal mouse myeloma cells. Polyclonal as well as monoclonal antibody to HBsAg may be used as coating and/or detecting reagent.

The biotin-binding substance is preferably conjugated to a marker (label). Suitable markers are an enzyme (including an enzyme substrate, cosubstrate, coenzyme, cofactor etc.), fluorescent, radioactive and chemiluminescent labels. Lanthanide labels such as europium, terbium, and samarium chelates may be used. A preferred marker is horseradish peroxidase.

In case an enzyme marker is employed, the measuring operation (step d) includes adding a substrate that develops a color that is measured and taken as an indication of the presence and amount of analyte in the sample.

The solid support employed in the invention are those ones commonly used in heterogeneous immunoassays. Thus the solid support could have different forms such as beads,

sheets, pads, wells of microtiter plates etc. The support may be porous or non-porous. It may consist of polystyrene, a polysaccharide, nylon, nitrocellulose, polypropylene etc. The artisan will know the material which is compatible with a certain physical form. The entities coated on the support may be bound thereto merely by physical adsorption or covalent attachment. The linkage between the support and the coated entity shall resist normal washing procedures applicable to heterogeneous immunoassays.

The reaction conditions for forming the complexes on the solid support are well known. Normally the medium is aqueous, the temperature 0-40°C, preferably 15-40°C, and the pH within the range 4-9. The medium may contain detergents and buffering components for stabilizing the pH.

In connection with the development work for the present invention, it has been realized that the non-specific binding in testing for anti-HIV antibodies by the use of the antigen:antibody:antigen-complex can be reduced by forming the complex in the presence of effective amount of an inhibitor for the interaction between an HIV envelope protein (e.g. gp120 for HIV 1 and gp105 for HIV 2) and the corresponding cell receptor (CD4). The amount is effective in the sense that it reduces the non-specific binding by preventing binding between the antigen coated on the support and antigen in the detecting reagent via HIV antigen receptors present as impurities. The addition of the inhibitor can completely or partially replace the use of antigens derived from different sources in antibody assays of the antigen:antibody:antigen-format.

This type of inhibitor has previously been suggested for the blocking of HIV-infection of CD4 target cells. See for instance Eric De Clercq (6th International Conference on AIDS, 21st June, 1990, San Fransisco). Parish C.R. et al. (J. Immunol. 145(1990)1188) have examined a number of sulfated polyanions for their ability to block anti-CD4 mAb binding. On CD4 they found a polyanion binding site that was clearly distinct but closely associated with the gp120 binding region of CD4. Generally speaking the known inhibitors are

polyanionic, in particular polysulfated or polysulfonated (i.e. exhibiting $-SO_3^-$ groups), polymers optionally containing a plurality of OH-groups. Suitable polymers are preferably soluble in aqueous media. Specific examples are sulfated polysaccharides, such as dextran sulfate, heparin, pentosan sulfate, fucoidan, and the carrageenans, and polyvinyl alcohol sulfate, and polyanethole sulfonate. The reduction of non-specific binding will have a positive effect on the specificity as well as on the sensitivity.

In order to have a good test, when using the above-mentioned inhibitor, normal optimization with respect to type, molecular weight, substitution degree etc. shall therefore always take place. For instance if the test simultaneously shall detect HBsAg with good sensitivity it might be necessary not to increase the inhibitor concentration too much. For an anti-HIV antibody test the concentration of dextran sulfate is recommended to be within 0.01-0.14%(w/w) or a concentration giving the equivalent or better effect if other CD4-gp120 HIV envelope inhibitors are used. In a combination test like the present invention the upper limit should in many cases be lowered, e.g. down to 0.10 %.

The invention is particularly useful for screening blood, plasma and serum, or their derivatives. However, samples derived from other biological fluids containing antibodies and infective agents may also be tested. For instance saliva, feces, urine, tissue culture fluids and other body or cell line fluids.

According to one aspect of the invention, a kit is provided for the detection of a first antigen or an antibody to a second antigen. The kit is comprised of a solid support coated with the second antigen and with an antibody to the first antigen, and detecting reagents for the first antigen and for antibody to the second antigen. Both the coated entity and the detecting reagent may have the specific modes specified above that particularly relates to HBsAg as the first antigen and HIV antigen as the second antigen. The kit may contain the detecting reagents in the form of an aqueous

suspension optionally containing buffer components or in the form of a lyophilized or spray-dried powder.

The aspects of the invention are more closely defined in the appending claims. The following examples (1-6) are provided to illustrate the invention but should not be interpreted as limiting its scope.

EXPERIMENTAL PART

METHODS:

Biotinylations: All biotinylations were performed by standard techniques, previously described by Guesdon et al. (J. Histochemistry 27(1979)p.1131-), using biotinyl N-hydroxysuccinimid (BNHS) (CalBiochem, San Diego, CA, U.S.A.).

Coating of microtiter plates: Polystyrene microtiter plates were coated with purified HIV antigen and mouse monoclonal antibody to HBsAg by a three step process. First, monoclonal antibody to HBsAg was coated in phosphate buffer, 0.1 M, pH 7.2. After incubating 12-16 hours at 4°C, the coating solution was aspirated off and purified HIV antigen was coated in a carbonate buffer, 0.1 M, pH 9.6. Plates were again incubated for 12-16 hours followed by aspiration. In the final step, plates were blocked to prevent non-specific adsorption of immunoglobulins and other serum proteins. The blocking buffer consisted of 0.1 M Tris, pH 7.4 with 0.7% bovine serum albumin, 5% sucrose, 0.1% (w/v) Tween 20 (Sigma, St Louis, Mo. U.S.A.) and preservatives.

Assaying protocol: 100/ul of each sample was pipetted into the wells of the microtiter plate. The plate was covered with a sealer and incubated at 37°C for 60 minutes. The plate was aspirated and washed five times with a wash buffer using 250-300/ul per well. 100/ul of the biotinylated reagents (Conjugate A) was added to each well of the microassay plate and a new plate sealer applied. The plate was then incubated at 37°C for 60 minutes. Again the plate was aspirated and washed five times as above. 100/ul goat anti-biotin conjugated to HRP (Conjugate B) was added to each well and a new plate sealer was applied. The plate was again incubated at 37°C for 60 minutes and aspirated and washed five times.

100/ul of substrate (OPD in excess) was added to each well and the plates were incubated at room temperature in the dark for 30 minutes. 100/ul of stop solution containing 2 N sulfuric acid was added and the developed color (absorbance) read at 492 nm within 60 minutes using a reference wavelength of 600-620 nm.

REAGENTS:

Anti HBsAg antibodies:

Monoclonal antibody to HBsAg was purchased from Sorin (Italy).

Polyclonal antibody to HBsAg from hyper-immunized goats was purified by standard techniques and biotinylated.

HIV antigens:

The HIV antigen used for coating of microtiter plates (HIV antigen) was derived from an HIV-1 infected H9 cell lysate purified by ultracentrifugation. For use in the detecting reagent the HIV-1 purified lysate was further purified prior to biotinylation by passage through a Sepharose anti-H9 affinity column. Unbound HIV-1 antigen was collected and biotinylated for use in Conjugate A below. The final product demonstrated a higher specific HIV-1 activity, relative to total protein, than prior to affinity purification. The Sepharose anti-H9 affinity column was prepared by covalently attaching goat antibody directed against human H-9 cellular proteins to CNBr activated Sepharose (Pharmacia AB, Uppsala, Sweden). The antibody was prepared by immunizing goats with an immunogen derived from uninfected human H-9 cells. The goats developed an antibody titer to uninfected H-9 cellular proteins, as well as to components that may remain from the H-9 cell culture media. The HIV antigen thus obtained was almost completely lacking H-9 proteins, and as an unavoidable consequence it had also been depleted of gp41, gp120, and gp160 envelope protein determinants

HIV antigen used as detecting reagent: In Examples 1 and 3-6 biotinylated HIV antigen from HIV-1 infected H9 cells mixed with biotinylated recombinant HIV gp160 envelope antigen (prepared in insect cells, Repligen Corp., Cambridge,

Mass., U.S.A.) was employed as a detecting reagent for antibody specific for HIV antigen. In Example 2, three different detecting reagents for HIV antigen were compared.

The biotinylated antibody to HBsAg and the biotinylated HIV antigen were used as a mixture (Conjugate A) dissolved in an aqueous medium containing 0.1 M Tris, 0.15 M sodium chloride, 0.025% Tween 20, 0.04% dextran sulfate, 5% bovine serum albumin (BSA), 0.5% 4-dimethylaminoantipyrin (DAP) as an antioxidant, 20% normal goat serum and 5% normal human serum.

Anti-biotin antibody:

Goat anti-biotin antibody labeled with horseradish peroxidase (Conjugate B) was purchased from Zymed Corp., South Fransisco, CA., U.S.A., and complexed with biotin bound to the plate during the assay. This antibody was used in a Tris buffered diluent of the same composition as given in the preceding paragraph.

HRP substrate: orto-phenylenediamine (OPD) dissolved in a buffer containing potassium phosphate, sodium citrate and hydrogen peroxide, pH 5.0.

Wash Buffer: Saline buffered with sodium phosphate and containing a surfactant.

Dextran sulfate: Purchased from Sigma Co., St. Louis, Mo., U.S.A.

EXAMPLE 1

The combination assay of the invention was compared with the Pharmacia HBsAg ELISA assay (Pharmacia Diagnostics Inc. Columbia, Md., U.S.A.) having specificity for HBsAg. Assay performance was compared utilizing two fold dilutions of a serum sample containing a known amount of the HBsAg analyte, but negative with respect to antibody to HIV. The measured absorbances are shown below in table 1. The commercially available Pharmacia HBsAg ELISA assay utilizes a monoclonal antibody to HBsAg on a microtiter plate well to capture HBsAg in the sample. A single conjugate containing a polyclonal antibody (goat) to HBsAg, chemically coupled to horseradish

peroxidase enzyme, is then utilized as a detection reagent to quantitate captured HBsAg in the sample.

TABLE 1.
PHARMACIA HBsAg ELISA vs PHARMACIA HIV/hep COMBI ELISA (invention) WITH TWO-FOLD DILUTIONS OF AN HBsAg POSITIVE SAMPLE (PAUL ERLICH INSTITUTE HBsAG STANDARD)

HBsAg Conc ng/mL	Absorbance HBsAg ELISA	Absorbance invention
2.50	2.101	2.530
1.25	0.909	1.646
0.62	0.631	0.824
0.31	0.205	0.365
0.16	0.121	0.269
0.00	0.030	0.045

The data in Table 1 demonstrate that absorbances obtained with the assay of the present invention were consistently greater than those obtained with Pharmacia HBsAg ELISA. Absorbances were consistently greater for all HBsAg concentrations tested, from 2.5 ng/mL to 0.16 ng/mL. This indicates that the assay of the invention is comparable or superior in sensitivity in detection of HBsAg in these samples, compared with the commercial HBsAg ELISA assay.

EXAMPLE 2:

Three assay formats were evaluated, differing in the detecting reagents employed. HIV antigen derived from an infected human H9 T-cell line was coated on microtiter plates for evaluation of all three formats. The detecting reagents were (a) biotinylated H9 cell derived HIV antigen (i.e. the same as used for coating the plate), (b) biotinylated HIV antigen derived from the HIV infected T-cell line MOLT 3, and (c) biotinylated recombinant HIV antigen (gp160 envelope protein, purchased from Repligen Corporation, Cambrigde, Mass. U.S.A., and produced in the insect cell line, Spodoptera frugiperda. Two known positive samples were tested. One positive sample demonstrated all the major bands on the western blot, e.g. p18, p24, p32, gp41, p51, p55, p66, gp120 and gp160. The other positive sample contained low levels of antibody to p24 as evidenced by a marked absence of

the p24 band on the western blot. This is generally explained on the basis of increased production of p24 antigen in advanced AIDS patiens and the resultant formation of p24 antigen:antibody complexes. Antibody complexed with excess p24 antigen is unavailable to bind to p24 antigen on the western blot; hence, the absence of a p24 band. In addition, six HIV negative samples were tested which were known to elicit a high non-specific signal in the Pharmacia VIRGO™ HIV-1 ELISA assay. Results are presented in Table 2 below. Values are given in units of absorbance at 492 nm wavelength.

TABLE 2:

	H9 Conjugate	MOLT 3 Conjugate	Recombinant Conjugate
Positive Sample	1.308	1.189	1.200
Positive Sample Low p24 Antibody	0.963	0.666	0.966
Negative Sample 1	0.619	0.029	0.043
Negative Sample 2	0.663	0.024	0.031
Negative Sample 6	0.701	0.025	0.029
Negative Sample 7	0.754	0.025	0.025
Negative Sample 8	0.826	0.024	0.031
Negative Sample 9	0.668	0.028	0.082

The two known positive samples were strongly reactive using all three detecting reagents. The "problem" negative samples, however, gave a markedly reduced non-specific signal where the MOLT-3 derived biotinylated HIV antigen or the biotinylated recombinant HIV antigen was employed as the detecting reagent, as compared with the biotinylated H9 derived HIV antigen. This marked drop in "noise" or non-specific background indicates a dramatic improvement in specificity as a result of the invention.

EXAMPLE 3:

The assay of the invention was compared with the Pharmacia HBsAg ELISA assay (see example 1). Samples tested

consisted of a release panel of diluted human sera known to be reactive for the presence of HBsAg (1-5, 7-9, 11-13) or known ot be non-reactive (6,10). The measured absorbance listed in Table 3 demonstrate that the assay of the invention has a sensitivity that is comparable or superior in detecting HBsAg in these samples. Samples 6 and 10 which are negative for HBsAg are non-reactive in the combination assay of the invention.

TABLE 3:

Sample No.	Absorbance Pharmacia HBSAg ELISA	Absorbance Combination Assay
1	2.556	2.919
2	2.303	2.735
3	2.558	3.203
4	2.825	2.977
5	2.951	2.977
6	0.026	0.065
7	2.876	2.789
8	0.271	0.427
9	0.090	0.139
10	0.055	0.068
11	2.852	2.647
12	0.200	0.220
13	3.177	2.893

EXAMPLE 4

The combination assay of the invention was compared with the Abbott HIV-1 EIA test (Abbott Laboratories, N. Chicago, IL. U.S.A.) to evaluate sensitivity. The Abbott HIV-1 EIA assay is a standard "sandwich" assay utilizing H9 cell derived HIV antigen coated on a 1/4" polystyrene bead to capture HIV antibody in the sample. Captured antibody (if present) is detected by the use of an enzyme conjugate consisting of goat anti-human IgG coupled to peroxidase

enzyme. A strong positive human serum sample was diluted serially (1:3:9:27:81:243:729:2187) in a negative serum pool and each dilution was tested with both assays. The absorbances measured are given in Table 5. The results indicate that the combination assay of the invention is more sensitive than the commercially available Abbott HIV-1 EIA test in the measurement of low levels of HIV-1 antibody in a typical HIV positive human sera.

TABLE 4

COMPARISON OF THE PHARMACIA HIV/HEP COMBI ASSAY WITH THE ABBOTT HIV-1 EIA ASSAY IN SERIAL DILUTIONS

Dilutions	Absorbances of Pharmacia HIV/Hep Combi ELISA Assay	Absorbances of Abbott HIV-1 EIA Assay
0	3.4	3.4
1:3	3.2	3.3
1:9	3.2	3.0
1:27	3.2	2.4
1:81	2.4	1.2
1:243	1.2	0.6
1:729	0.8	0.2
1:2187	0.6	0.1

EXAMPLE 5

The assay of the invention was compared with the Pharmacia HIV-1 ELISA assay (Pharmacia Diagnostics, Columbia, Md. U.S.A.), that is a comercially available test for antibody to HIV. The assay is a standard "sandwich" format utilizing H9 cell derived HIV antigen coated on a microtiter plate to capture HIV antibody in the sample. Captured antibody (if present) is detected by the use of an enzyme conjugate consisting of (goat) anti-human IgG (H + L chains) polyclonal antibody, covalently coupled to peroxidase enzyme. A strong positive human serum sample was diluted serially (1:3:9:27:81:243:729:2187) in a negative serum pool and each dilution was tested with both assays. The absorbances measured are given in Table 6. The results indicate that the combination assay of the invention is more sensitive than the commercially available Pharmacia HIV-1 ELISA test in the

measurement of low levels of HIV-1 antibody in a typical HIV positive human sera.

TABLE 5

COMPARISON OF THE PHARMACIA HIV/HEP COMBI ASSAY WITH THE PHARMACIA HIV-1 ELISA ASSAY IN SERIAL DILUTIONS

Dilutions	Absorbances of Pharmacia HIV/Hep Combi ELISA Assay	Absorbances of Pharmacia HIV-1 ELISA ASSAY
0	3.2	3.4
1:3	3.1	3.3
1:9	3.1	3.3
1:27	3.2	3.2
1:81	3.1	2.1
1:243	3.0	1.3
1.729	2.9	0.3
1:2187	2.0	0.2

EXAMPLE 6

The assay of the invention was compared with the Pharmacia HIV-1 ELISA assay (see example 5). The goal of the experiment was to study various samples that are positive for antibody to HIV and determine the highest dilution in which the antibody could be detected. The highest dilution detectable for each sample is given in Table 6. The data clearly show that the assay of the invention was able to detect quantities of more diluted antibody than the commercial assay.

Table 6:

Detection of HIV-1 Antibody Dilutions

Sample No.	Combination Assay	Commercial Assay
1	10000	700
2	2200	700
3	2200	2200
4	20000	700
5	2200	700
6	2200	700
7	80	80
8	1000	100
9	1000	100
10	2000	1000
11	2000	1000
12	10000	2000
13	10000	100
14	5000	500

What is claimed is:

1. A combination immunoassay for determining the presence, absence, or amount of a first antigen and/or an antibody to a second antigen in a sample, which immunoassay comprises the steps:

a) contacting the sample with a solid support on which the second antigen and an antibody to the first antigen have been coated so that said first antigen and antibody to said second antigen, when present in the sample, will become bound to (complexed with) their immunological counterparts, respectively, coated on the solid support;

b) adding second antigen in the form of a detecting reagent for antibody bound by the second antigen coated on the solid support; the addition being performed under conditions allowing the formation of an immune complex between said added second antigen and said antibody specific to said second antigen bound to the solid support in step (a);

c) adding antibody to said first antigen in the form of a detecting reagent for first antigen bound by the antibody coated on the solid support; the addition being performed under conditions allowing the formation of an an immune complex between said added first antibody and first antigen bound to the solid support in step (a);

d) determining the presence, absence, or amount of the immune complex formed in step (b) and (c) in order to determine the presence, absence, or amount of the first antigen and/or the antibody to the second antigen in the sample,

wherein the second antigen coated on the solid support contains antigen that is derived from a first source and the second antigen used as a detecting reagent contains antigen that is derived from a second source, which first and second sources are different.

2. The immunoassay of claim 1 wherein the sources are two different cell lines expressing the antigen.

3. The immunoassay of claim 2 wherein the second antigen is a virus antigen and the cell lines are selected from the group consisting of (i) a cell line infected with the virus and (ii) a recombinant host cell line expressing the antigen.

4. The immunoassay of claim 3 wherein one of the cell lines is a recombinant cell line of a defined host while the other cell line is a recombinant cell line of a different host.

5. The immunoassay of claim 4 wherein one of the cell lines is an insect host cell line and the other cell line is an E. coli host cell line.

6. The immunoassay of claim 1 wherein the second antigen is HIV antigen and the first antigen is a hepatitis virus antigen.

7. The immunoassay of claim 6 wherein the first antigen is HBsAg.

8. The immunoassay of claim 1 wherein the second antigen and antibody to the first antigen added in step (b) and (c), respectively, are biotinylated, a biotin-binding substance is added in step (d) to complex with the biotinylated reagents bound to the solid support in step (b) and (c), and the presence, absence, or amount of the complex between biotin and the biotin-binding substance on the solid support is detected in order to determine the presence, absence, or amount of the first antigen and/or antibody to the second antibody.

9. The immunoassay of claim 8 wherein the biotin-binding substance added is anti-biotin antibody labeled with a marker.

10. A combination immunoassay for determining the presence, absence, or amount of a first antigen and/or an antibody to a second antigen in a sample, which immunoassay comprises the steps:

a) contacting the sample with a solid support on which the second antigen and antibody to the first antigen have been coated so that said first antigen and antibody to said second antigen, when present in the sample, will become bound to (complexed with) their immunological counterparts, respectively, coated on the solid support;

b) adding biotinylated second antigen under conditions allowing the formation of an immune complex between said biotinylated second antigen and antibody specific to said second antigen bound to the solid support in step (a);

c) adding biotinylated antibody to said first antigen under conditions allowing the formation of an an immune complex between said biotinylated antibody and said first antigen bound to the solid support in step (a);

d) determining the presence, absence, or amount of the immune complex formed in step (b) and (c) by

d1) addition of a biotin-binding substance labeled with a marker to complex with the biotinylated reagents bound to the solid support in step (b) and (c), and

d2)detecting the presence, absence, or amount of the complex between biotin and the biotin-binding substance on the solid support in order to determine the presence, absence, or amount of the first antigen and/or antibody to the second antibody.

11. The immunoassay of claim 10 wherein the second antigen coated on the solid support contains antigen that is derived from a first source and the second antigen used as a detecting reagent contains antigen that is derived from a second source, which first and second sources are different.

12. The immunoassay of claim 11 wherein the sources are two different cell lines expressing the antigen.

13. The immunoassay of claim 12 wherein the second antigen is a virus antigen and the cell lines are selected from the group consisting of (i) a cell line infected with the virus and (ii) a recombinant host cell line expressing the antigen.

14. The immunoassay of claim 13 wherein one of the cell lines is a recombinant cell line of a defined host while the other cell line is a recombinant cell line of a different host.

15. The immunoassay of claim 14 wherein one of the cell lines is an insect host cell line and the other cell line is an E. coli host cell line.

16. The immunoassay of claim 10 wherein the second antigen is HIV antigen and the first antigen is a hepatitis virus antigen.

17. The immunoassay of claim 16 wherein the first antigen is HBsAg.

18. The immunoassay of claim 10 wherein steps (b) and (c) are performed simultaneously.

19. The immunoassay of claim 1 wherein steps (b) and (c) are performed simultaneously

20. The immunoassay of claim 12 wherein the second antigen is HIV antigen and the cell lines are H9 and MOLT-3, respectively, infected with HIV.

21. The immunoassay of claim 3 wherein the second antigen is HIV antigen and the cell lines are H9 and MOLT-3, respectively, infected with the HIV.

22. The immunoassay of claim 6 wherein the HIV antigen coated on the support or used as the detecting reagent is gp160.

23. The immunoassay of claim 4 wherein the second antigen is HIV antigen and one of the recombinant cell lines expresses gp160.

24. The immunoassay of claim 1 wherein the second antigen is HIV antigen that is a mixture of HIV antigens from two or more sources, and the first antigen is HBsAg.

25. The immunoassay of claim 10 wherein the second antigen is HIV antigen that is a mixture of HIV antigens from two or more sources, and the first antigen is HBsAg.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/06675

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

Accerding to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): .C12Q 1/70

U.S. Cl.: 435/5

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S. Cl.	435/5

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X/Y	US, A, 4,520,113 (Gallo et al.) 28 May 1985, see entire document.	1-25 1-25
Y,P	US, A, 4,959,323 (Acs et al.) 25 September 1990, see entire document.	1-25
Y	US, A, 4,629,783, (Cosand) 16 December 1986, see entire document.	1-25

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
10 December 1990	12 FEB 1991
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	Bradley L. Sisson

Form PCT/ISA/210 (second sheet) (May 1986)